

DATA EVALUATION RECORD
CHRONIC (MULTIPLE DOSE) TOXICITY TESTS WITH THE HONEY BEE LARVAE
NON-GUIDELINE

1. **CHEMICAL:** Dicamba PC Code No.: 029801

2. **TEST MATERIAL:** Dicamba (BAS 183 H) Purity: 93.9%

3. **CITATION**

Authors:	Kleebaum, K.
Title:	Repeated exposure of honey bee (<i>Apis mellifera</i>) larvae to BAS 183 H (Dicamba) under laboratory conditions
Study Completion Date:	October 8, 2018
Laboratory:	BioChem agrar GmbH Gerichshain, Germany
Sponsor:	BASF SE Ludwigshafen, Germany
Laboratory Report ID:	18 48 BLC 0033
MRID:	50784602
DP Barcode:	451249

4. **REVIEWED BY:** Rebecca L. Bryan, Staff Scientist, CDM/CSS-Dynamac JV

Signature: 

Date: 4/23/2019

APPROVED BY: Moncie V. Wright, Ph.D., Environmental Scientist, CDM/CSS-Dynamac JV

Signature: 

Date: 6/17/2019

(Stats re-do & DER updates completed on 10/21/2019)

5. **Reviewed by:** Michael Wagman, Senior Scientist, OPP/EFED/ERB2

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Reviewed by: Edward Odenkirchen, Ph.D., Senior Science Advisor, OPP/EFED/IO

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6. **DISCLAIMER:** This Data Evaluation Record may have been altered by the Environmental Fate and Effects Division subsequent to signing by CDM/CSS-Dynamac JV personnel. The CDM/CSS-Dynamac Joint Venture role does not include establishing Agency policies.

7. **STUDY PARAMETERS:**

Test Species and strain: Honey bees (*Apis mellifera* L.)

Age of Test Organisms at Test Initiation: 1st instar larvae; 1 day old

Exposure Duration: 4 days of exposure (as part of 22-day test)

8. **CONCLUSIONS:**

Individual synchronized honey bee (*Apis mellifera*) larvae (first instar) were exposed *in vitro* to Dicamba on Days 3 through 6 of the study at the nominal concentrations reported by the study author and the nominal daily doses calculated by the reviewer (cumulative doses were divided by the number of days of exposure) reported in the table below. The study author calculated the mean-measured concentrations from the analytical results of the test item measured in royal jelly diet samples. The reviewer used the % recoveries of the test item to calculate the measured daily dietary doses. The measured concentrations/doses are summarized in the table below:

Nominal Concentrations (mg ai/kg diet)	Mean Measured Diet Concentrations (mg ai/kg diet)	Nominal Daily Doses (µg ai/larva/day)	Measured Daily Dietary Doses (µg ai/larva/day)
39.81	35.07	1.6	1.3
79.61	66.90	3.2	2.6
159.2	129.7	6.3	5.1
318.4	260.9	13	10
636.9	540.0	25	21

A negative (untreated diet) control was included in the 22-day study. Dimethoate technical was used as a reference toxicant at 48 µg/g diet corresponding to 7.6 µg ai/larva. All groups consisted of 36 larvae total per group; each larva was contained within a polystyrene grafting cell that was within a 48-well cell culture plate. Each of 3 colonies was individually considered a replicate.

On Day 8, larval mortality averaged 6% in the negative control, as compared to mortality ranging from 6 to 14% in the groups exposed to the test material. By Day 15, mortality averaged 14% in the control, and ranged from 17 to 42% in the exposed groups. Emergence averaged 81% in the control, as compared to emergence ranging from 22 to 78% in the

groups exposed to the test material.

Significant effects were observed for pupal mortality and adult emergence during the 22-day study. The NOAEC and EC₅₀ were 129.7 and 247 mg ai/kg diet, respectively (corresponding to a NOAEL and ED₅₀ of 5.1 and 9.6 µg ai/larva/day, respectively).

The study **is scientifically sound and is consistent with the OECD Guidance Document** for measuring chronic (repeat dose) toxicity to honey bee larvae. The study is classified as **acceptable**

	Mortality (Day 8)	Mortality (Day 15)	Adult Emergence
Diet Concentration (mg ai/kg diet)	LC ₅₀ : >540.0 95% CI: N/A Slope: N/A NOAEC: 540.0 LOAEC: >540.0	LC ₅₀ : >540 95% CI: -N/A Slope: N/A NOAEC: 129.7 LOAEC: 260.9	EC ₅₀ : 247 95% CI: 171 - 426 Slope: 1.2 (0.8 – 1.7) NOAEC: 129.7 LOAEC: 260.9
Dietary Dose (µg ai/larva/day)	LD ₅₀ : >21 95% CI: N/A Slope: N/A NOAEL: 21 LOAEL: >21	LD ₅₀ : >21 95% CI: N/A Slope: N/A NOAEL: 5.1 LOAEL: 10	ED ₅₀ : 9.6 95% CI: 6.6 - 17 Slope: 1.2 (0.7 – 1.7) NOAEL: 5.1 LOAEL: 10

9. ADEQUACY OF THE STUDY:

A. Classification: This study **is scientifically sound** and is classified as **acceptable**.

B. Rationale:

C. Reparability:

10. GUIDELINE DEVIATIONS: This study is based on Guidance Document on Honey Bee Larval Toxicity Test following Repeated Exposure, Series on Testing and Assessment, No. 239, OECD (2016) with adaptations. The following deviations from OECD 239 were noted by the reviewer:

- 1.) The larvae were used in the test were C-shaped, which is not in accordance with OECD guidance which highly recommends selecting larvae that have not yet formed a C shape.
- 2.) The % composition of the components of Diet A were not in accordance with OECD recommendations.
- 3.) No individual bee weight data were provided for the 36 bees in each control and treatment group. Per U.S. EPA "Honeybee Toxicity Testing Frequently Asked

Questions – August 8, 2018” the individual larva is considered the replicate on the basis of each larva being housed in a separate cell and receiving its own dose. In this study, a replicate was defined as a group of 12 larvae from each of 3 colonies (three replicates = three colonies).

These deviations **did not** impact the acceptability of this study.

- 11. SUBMISSION PURPOSE:** To determine the effects on mortality and sublethal effects of Dicamba on the honey bee (*A. mellifera* L.) larvae from chronic [multiple dose] exposure following the OECD Guidance Document for the purpose of chemical re-registration.

12. MATERIALS AND METHODS:

A. Test Material:	Dicamba (BAS 183 H)
Description:	Not reported
Lot No./Batch No.:	0002B01BA-251
Purity:	93.9%
IUPAC Name:	3,6-dichloro-2-methoxy-benzoic acid
CAS Name:	3,6-dichloro-2-methoxybenzoic acid
CAS Number:	1918-00-9
Stability of compound under test conditions:	The mean measured diet concentrations were 81 to 88% of nominal over the four-day exposure period.
Storage conditions of test chemical:	Room temperature (+5°C to +35°C)

Range finding test yes/no (if yes, describe): No range-finding test was reported.

Physicochemical properties of the Dicamba.

Parameter	Values	Comments
Molecular Weight	Not reported	
Water solubility at 20°C (mg/L)	Not reported	
Vapor pressure (torr, at 25°C)	Not reported	
Structure	Not reported	
Mean organic carbon partition coefficient K_{oc} (L/kg_{oc})	Not reported	
Log octanol-water partition coefficient Log K_{ow}	Not reported	

B. Test Organisms

Guideline Criteria	Reported Information	Comments
Species	Honey bee (<i>Apis mellifera</i> L.; subspecies Buckfast)	<i>OECD recommends European honey bee (Apis mellifera)</i>
Age at beginning of test Worker bees of uniform age.	First instar larvae (24-hr old)	<i>OECD recommends that on D1 of study, first instar (L1) synchronized larvae (i.e., larvae of the same age) are taken from comb of three colonies.</i>
Source	Bees collected from three in-house laboratory colonies.	<i>OECD recommends larvae are collected from three different colonies.</i>
Were bees from disease-free colonies?	Yes. All larvae used in the test derived from healthy (free of clinical symptoms of any disease) and queen-right bee colonies. The larvae were taken from hives that had not received treatments with chemical substances for at least one month.	<i>OECD recommends that colonies used to obtain larvae should be adequately fed, health (i.e., as far as disease- and parasite-free), with a known history and physiological status.</i>
Were bees kept in conditions conforming to proper cultural practices?	Not reported.	

C. Test System

Guideline Criteria	Reported Information	Comments
Test Chambers	Crystal polystyrene grafting cells (CNE Nicotplast, internal diameter 9 mm) were placed in 48 well plates.	<i>OECD recommends 48-well plate with each well containing a crystal polystyrene grafting cell.</i>
Temperature during exposure	34.0-34.8°C	<i>OECD recommends incubator at 34 – 35°C.</i>

Guideline Criteria	Reported Information	Comments
		<i>Deviations may occur but temperature should not be lower than 23°C or higher than 40°C; deviations not last more than 15 minutes once every 24 hrs.</i>
Relative humidity during exposure	Day 1-8: 93-96% Day 8-15: 82-85% Day 15-22: 58-60%	<i>OECD recommends use of K₂SO₄ to maintain water saturated atmosphere.</i>
Lighting	Constant darkness except during handling and assessments where diffuse artificial light was used.	<i>OECD recommends that plates should be maintained in darkness.</i>
Feeding	<p>Each larva was fed once a day (except on D2) with a standardized amount of artificial diet of 20 µL untreated diet A on Day 1 (D1), 20 µL treated/untreated diet B on Day 3 (D3), and 30, 40, and 50 µL treated/untreated diet C on Days 4, 5, and 6 (D4-6), respectively.</p> <p>The aqueous yeast/sugar solutions were prepared prior to the study and stored frozen ($\leq -18^{\circ}\text{C}$) before use. The sugar solution was mixed with royal jelly every day before feeding, and each larva was fed separately using a sterile pipette. The food drop was placed next to the larvae to avoid drowning. Before feeding, the final diets were tempered up to about 34.5°C. During feeding, the culture plate was placed on a warming plate to prevent the larvae from cooling down.</p> <p><u>Diet A</u>: 44.25% weight of fresh royal jelly + 55.75% weight of an aqueous solution containing 1.61% weight of yeast extract, 9.5% weight of glucose and 9.5% weight of fructose</p>	<p><i>OECD recommends that all larvae are fed once a day. Volume of diet is adjusted each day. Additional food should be added to the cell even if previous allocation has not been totally consumed. Presence of uneaten food at termination of test should be reported.</i></p> <p><i>OECD recommends:</i> <i>Diet A (D1): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.</i> <i>Diet B (D3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.</i></p>

Guideline Criteria	Reported Information	Comments
	<p><u>Diet B</u>: 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.</p> <p><u>Diet C</u>: 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.</p>	<i>Diet C (from D4 to D6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.</i>

D. Test Design

Guideline Criteria	Reported Information	Comments
Nominal dosage levels tested	<p>Diet concentrations: 0 (negative control), 39.81, 79.61, 159.2, 318.4, and 636.9 mg ai/kg diet</p> <p>Cumulative dietary doses: 0 (negative control), 6.3, 12.6, 25, 50, and 101 µg ai/larva)</p> <p>Daily dietary doses: 0 (negative control), 1.6, 3.2, 6.3, 13, and 25 µg ai/larva/day</p>	<p>OECD recommends 5 treatments of increasing test concentrations.</p> <p>Alternatively, when a limit test is performed, a single dose of 100 µg ai/larva or the maximum achievable solubility (whichever is lower).</p>
Measured test concentrations	<p><0.031 (<LOD, control), 35.07, 66.90, 129.7, 260.9, and 540.0 mg ai/kg diet</p> <p>1.3, 2.6, 5.1, 10, and 21 µg ai/larva/day</p>	The measured daily doses were reviewer-calculated based on mean measured percent recoveries and 4 days of exposure.

Guideline Criteria	Reported Information	Comments
Number of bees exposed per dosage level	36 larvae per treatment group.	<i>OECD recommends minimum of 12 larvae from each of 3 colonies allocated on the same plate to each treatment, i.e., minimum of 36 larvae per treatment.</i>
Other experimental design information	On Day 8, the pre-pupae larvae were transferred to new pupal plates.	<i>OECD recommends that newly hatched larvae are selected that have not yet formed a "C" shape and randomizing the allocation of larvae into the plates for each colony. On Day 1, larva is deposited in cell containing 20 µL diet.</i>
Bees randomly or impartially assigned to test groups	All plates used in the study were randomized.	<i>OECD recommends that each group of a minimum of 12 larvae from each of the three colonies is considered a replicate for a given treatment level and identified as such on the microplate.</i>
Control	Negative control: untreated diet 36 larvae total	larval control mortality was 5.6%_by D8 and 19.4% at test termination. <i>OECD recommends 12 larvae x 3 colonies=36 larvae minimum and that control mortality from D4 to D7 should be ≤15%, and ≤30% at test termination</i>
Solvent control	N/A	<i>OECD recommends maximum of 5%.</i>

Guideline Criteria	Reported Information	Comments
Reference Toxicant	The reference item, dimethoate, was tested at 48 mg ai/kg diet (equivalent to 7.6 µg ai/larva). 36 larvae total	Mortality was 89% by D8. <i>OECD recommends technical grade dimethoate at dose of 8.8 ± 0.5 µg a.i./larva. Mortality should be $\geq 50\%$ at D7 for toxic reference.</i>
Total observation period and frequency of interim observations	Number of dead larvae (an immobile larva or one which did not react to contact was noted as dead) on Days 4 to 8 (larvae) and Day 15 (pupae). Total mortality determined from Day 15 to 22 mortality. On Day 22 (final assessment), the bees which emerged successfully were counted. On Day 8, any larger amounts of unconsumed food and sublethal effects (discolorations, abnormal behavior, and/or substantially undersized larvae) were determined.	To correct for effects observed in the treatment group caused by background mortality, any calculations were performed using "mortality" instead of "adult emergence". <i>OECD recommends that following chemical exposure on D4, mortalities are checked at time of feeding on D5, D6 and D7 (test termination). Other observations including presence of uneaten food on D7 should be qualitatively reported.</i>

13. REPORTED RESULTS:

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statements were included in the report?	Yes, study conducted in compliance with U.S EPA (40 CFR, Parts 160 and 792) and OECD (1997) GLP regulations, with the exception that recognized differences exist between the GLP Principles/Standards of OECD and the Principles/Standards of FIFRA and TSCA.
Observed adverse effects on bees at respective dosages	Yes; details provided in the Mortality and Observations section.

Guideline Criteria	Reported Information
Control and Solvent Control Mortality	Negative control: <u>Day 8 mortality</u> : 6% <u>Day 15 mortality</u> : 14% <u>Day 22 mortality</u> : 19%
Were raw data included?	Yes
Analytical Analysis?	Diet samples were analyzed by liquid chromatography with mass spectrometry (LC-MS/MS).

Mortality and Observations:

On Day 8, larval mortality averaged 6% in the negative control, as compared to mortality ranging from 6 to 14% in the groups exposed to the test material (Table 1). By Day 15, mortality averaged 14% in the control, and ranged from 17 to 42% in the exposed groups (Table 2). Emergence averaged 81% in the control, as compared to emergence ranging from 22 to 78% in the groups exposed to the test material (Table 2).

The positive control (dimethoate) caused an increase in larval mortality (89%) at 48 mg ai/kg diet (equivalent to 7.6 µg ai/larva) compared to the negative control.

Table 1. Cumulative honey bee larval mortality data after repeated dietary exposure (dose).^a

Mean-Measured Diet Concentration, mg ai/kg (measured dietary dose, µg ai/larva/day)	Number Exposed	Day 4 mortality (%)	Day 5 mortality (%)	Day 6 mortality (%)	Day 7 mortality (%)	Day 8 mortality (%)
Negative Control	36	0	0	0	0	6
35.07 (1.3)	36	0	3	3	3	6
66.90 (2.6)	36	0	0	3	3	6
129.7 (5.1)	36	0	0	6	6	14
260.9 (10)	36	0	0	3	8	14*
540.0 (21)	36	0	0	3	3	14*
Dimethoate 48 (7.6 µg ai/larva)	36	6	42	72	75	89

^a Data were obtained from Appendix 4 on page 31 of the study report, and percent data were reviewer-calculated for Days 4 to 7.

* Significantly increased mortality compared to the control.

Table 2. Cumulative honey bee pupal mortality, adult emergence, and weight at emergence data after repeated dietary exposure (dose).^a

Mean Measured Diet Concentration, mg ai/kg (measured dietary dose, µg ai/larva/day)	Day 15 mortality (%)	Day 22 mortality (%)	Day 22 Adult Emergence (%)	Day 22 Adult Weight at Emergence
Negative Control	14	19	81	NR

Mean Measured Diet Concentration, mg ai/kg (measured dietary dose, µg ai/larva/day)	Day 15 mortality (%)	Day 22 mortality (%)	Day 22 Adult Emergence (%)	Day 22 Adult Weight at Emergence
35.07 (1.3)	17	22	78	NR
66.90 (2.6)	19	22	78	NR
129.7 (5.1)	25	28	72	NR
260.9 (10)	39	42*	58*	NR
540.0 (21)	42	78*	22*	NR
Dimethoate 48 (7.6 µg ai/larva)	94	94	6	NR

^a Data obtained from Appendix 4 on page 31 of the study report.

* Significantly different compared to the control.

NR Not reported

Reported Statistical Analysis

For each concentration, the corrected mortality was calculated according to Abbott (1925) modified by Schneider Orelli (1947) following a formula.

For statistical evaluation of NOAEC/NOAEL values, the Step-down (Rao-Scott) Cochran-Armitage Test was used for the Day 8 and Day 22 data. The accepted significance level was $p \leq 0.05$ (one-sided greater). The Day 8 LD/LC₅₀ values were determined with the Weibull analysis with linear maximum likelihood regression. The Day 22 ED/EC₅₀ calculations were performed with the Logit analysis with linear maximum likelihood regression. Mortality of control and reference item on Days 8 and 22 were compared using the Fisher's Exact Binominal Test (alpha = 0.05, one-sided greater). The statistical calculations were performed with the computer program ToxRatPro 3.2.1 (2015). The study author results are based on nominal diet concentrations and nominal cumulative doses.

Treatment groups	Larval Mortality (Day 8)	Adult Emergence
Nominal Test Concentration, mg ai/kg diet	LC ₅₀ : >637 95% CI: N/A Slope: N/A NOAEC: 159 LOAEC: 318	EC ₅₀ : 446 95% CI: 350-568 Slope: N/A NOAEC: 159 LOAEC: 318
Nominal Cumulative Dietary Dose, µg ai/larva	LD ₅₀ : >101 95% CI: N/A Slope: N/A NOAEL: 25 LOAEL: 50	ED ₅₀ : 70.4 95% CI: 55.2-89.7 Slope: N/A NOAEL: 25 LOAEL: 50

Reviewer's Statistical Analysis

The reviewer analyzed the mortality and emergence data using CETIS statistical software version 1.9.5.3 with database backend settings implemented by EFED on 7/25/17.

The mean-measured diet concentrations (mg ai/kg diet) and measured daily dietary doses (µg ai/larva/day) were used for the analyses and are represented in CETIS as two separate test records named with the MRID number and followed by the acronyms “dc” (= diet concentration), and “dd” (= dietary dose).

Larval mortality was analyzed using Fisher's Exact Test with a Bonferroni-Holm correction, and pupal mortality and emergence were analyzed using the Cochran Armitage Trend Test.

Due to a lack of significant effects for larval mortality and a maximum effect of 9%, relative to the negative controls, the reviewer empirically estimated the LC/LD₅₀ values as being greater than the highest test concentration/dose. The reviewer used linear regression to determine the LC/LD/EC/ED₅₀ values for pupal mortality and emergence. Day 15 values should be interpreted with caution, as they are extrapolated beyond the highest measured dose/concentration and the upper 95% confidence limit is unreasonably large so confers less reliability to the LD/LC₅₀.

All analyses were conducted at $\alpha = 0.05$ unless specified otherwise.

	Mortality (Day 8)	Mortality (Day 15)	Adult Emergence
Diet Concentration (mg ai/kg diet)	LC ₅₀ : >540.0 95% CI: N/A Slope: N/A NOAEC: 540.0 LOAEC: >540.0	LC ₅₀ : >540 95% CI: -N/A Slope: N/A NOAEC: 129.7 LOAEC: 260.9	EC ₅₀ : 247 95% CI: 171 - 426 Slope: 1.2 (0.8 – 1.7) NOAEC: 129.7 LOAEC: 260.9
Dietary Dose (µg ai/larva/day)	LD ₅₀ : >21 95% CI: N/A Slope: N/A NOAEL: 21 LOAEL: >21	LD ₅₀ : >21 95% CI: -N/A Slope: N/A NOAEL: 5.1 LOAEL: 10	ED ₅₀ : 9.6 95% CI: 6.6 - 17 Slope: 1.2 (0.7 – 1.7) NOAEL: 5.1 LOAEL: 10

14. REVIEWER'S COMMENTS:

The reviewer's and the study author's NOAEC/NOAEL for larval mortality were not in agreement, likely because the reviewer used Fisher's Exact Test with a Bonferroni-Holm correction whereas the study author used the Step-down (Rao-Scott) Cochran-Armitage Test. The NOAEC/NOAEL for emergence were in agreement, but the reviewer's EC/ED₅₀ values were far lower than the study author's, which can be attributed to the reviewer using linear regression and the measured diet concentrations/daily dietary doses and the study author using the logit analysis with linear maximum likelihood regression and the nominal diet concentrations/cumulative doses. Additionally, the reviewer analyzed pupal mortality data. The reviewer's results are reported in the Conclusions and Reviewer's Conclusions sections of this DER. The OECD guidance document's validity criteria for negative control performance were met with larval and test termination below 15% and 30%, respectively. The validity criteria for the dimethoate positive control were also met with >50% mortality by D8.

The in-life phase of the test was conducted from August 13 to September 3, 2018.

15. REVIEWER'S CONCLUSIONS:

This study is **scientifically sound** and is classified as **acceptable**. Significant effects were observed for pupal mortality and adult emergence during the 22-day study. The NOAEC, LOAEC and EC₅₀ were 129.7, 260.9 and 247 mg ai/kg diet, respectively, corresponding to a NOAEL, LOAEL and ED₅₀ of 5.1, 10 and 9.6 µg ai/larva/day, respectively.

16. REFERENCES:

None, other than standard guidelines or methodologies.